Improving headspace-solid-phase microextraction of 3-isobutyl-2-methoxypyrazine by experimental design with regard to stable isotope dilution gas chromatography–mass spectrometric analysis of wine

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Abstract

To solve problems of sensitivity, repeatability and multi-step extraction related to 3-isobutyl-2-methoxypyrazine (IBMP) determination in wines, a simple method based on the novel combination of solid-phase microextraction and stable isotope dilution assay is presented. Among the parameters that affect this type of extraction, five of them have been optimised since the other parameters have common values or do not require optimisation (e.g. addition of sodium chloride at saturated concentration) and so were fixed. Vial volume, sample volume/vial volume ratio, pH, adsorption time and temperature have been optimised by means of two experimental designs. After extraction, quantification was performed by stable isotope dilution with gas chromatography-tandem mass spectrometry ([$^2H_2$]-IBMP as internal standard). The final procedure allowed quantification far below IBMP's sensory threshold (1 ng l^{-1} versus 15 ng l^{-1}) with a 4% standard deviation. This method has been applied to experimental Fer servadou wines. Comparison of IBMP contents confirmed the efficiency of some viticultural and enological techniques on the herbaceous flavour decrease, such as prior fermentation maceration at high temperature (70 °C) and the use of a reflective carpet on viticultural soil.

Keywords: Solid-phase microextraction; Stable isotope dilution; Experimental design; Wine; 3-Isobutyl-2-methoxypyrazine

1. Introduction

The Fer servadou grape variety (*Vitis vinifera cv. Fer servadou*) comes from south-west France. This variety belongs to the same family as Cabernet Sauvignon. Among its varietal key aroma compounds, 3-alkyl-2-methoxypyrazines have been identified, in particular 3-isobutyl-2-methoxypyrazine (IBMP), known for its contribution to the green bell pepper odour. This compound is present at very low concentrations (10–50 ng l^{-1} in wines and up to 80 ng l^{-1} in grapes) but IBMP has a high odorant potency since its sensory threshold was reported to be 10 ng l^{-1} in red wines [1].

To diminish the IBMP concentration, wine makers evaluated different techniques based on thermolability and photosensitivity of IBMP [2], such as prior fermentation maceration at high temperature or the use of a reflective carpet on viticultural soil. A reliable analysis for IBMP is then needed for evaluating effects of those technical developments and also environmental factors.

Detection of volatiles at low concentrations (ng l^{-1}) requires an extraction-concentration step coupled to a very sensitive analytical technique. Several extraction-concentra-

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doi:10.1016/j.aca.2003.11.083

tion methods have been used: extraction by cation-exchange resin, liquid–liquid extraction [3] and a method based on headspace-solid-phase microextraction (HS-SPME) after concentration by evaporation [4]. All these extractions are multi-step, time-consuming and laborious. The analysis can be performed either by gas chromatography (GC) with nitrogen–phosphorus detection (NPD) (non-labelled internal standard) or by GC-mass spectrometry (MS) using deuterium labelled [$^2H_2$]-IBMP. Recently, an attempt to simplify the extraction step to only one, SPME, followed by GC-NPD for analysis encountered sensitivity and repeatability problems (coefficient of variation = 8–18%) [5]. To solve these problems and also to permit automation, we chose to combine SPME with stable isotope dilution GC. This type of calibration, using deuterium labelled [$^2H_2$]-IBMP as internal standard, is performed by GC-MS/MS.

This combination seems to be appropriate since the two molecules (target and standard) react in the most similar behaviour with the extraction fibre. After optimisation of the SPME conditions, the described method has been applied to wine samples.

2. Experimental

2.1. Reagents

3-Isobutyl-2-methoxypyrazine [24683-00-9] was supplied by Aldrich. The purity of the standard was >99%. The labelled [$^2H_2$]-IBMP was synthesized as described in [6]. Stock solutions of 1 g l^{-1} were prepared in RP Normapur absolute ethanol (Prolabo), and stored in the dark at +4 °C. To adjust the pH, potassium hydroxide (85% ACS reagent, Aldrich) at 200 g l^{-1} was used.

A wine model solution contained in 1 l of demineralised water 6 g of glycerol (Aldrich), 2.5 g of L-(+)-tartaric acid (Aldrich), 3 g of L-(+)-lactic acid (Fluka), 1 g of potassium phosphate (Aldrich), 11% absolute ethanol (Prolabo), pH adjusted to 3.5.

2.2. SPME procedure

An SPME manual device and 65 μm polydimethylsiloxane-divinylbenzene (PDMS-DVB) fibres were purchased from Supelco. To prevent contamination, each fibre was conditioned and cleaned before and after use by inserting it into the GC injector at the recommended temperature. A visual daily control is assumed (colour, coating state ...).

For optimisation, parameters were fixed as mentioned in the experimental design section and as described in Table 1.

The final procedure for calibration was as follows: 25 ml of a wine solution was adjusted to the optimum pH (6.6) with potassium hydroxide solution (200 g l^{-1}) and spiked with 10.5 ng l^{-1} [$^2H_2$]-IBMP. Ten millilitres of this solution was transferred into a 20 ml vial containing 3 g of sodium chloride and a magnetic stirrer. The vial was immediately capped with a PTFE-faced silicone septum/aluminium crimp cap. The extraction was performed in the headspace over 165 min at 27 °C, with constant stirring.

Table 1
SPME optimised parameters values for experimental design[a]

Parameter	Previous method for musts [7]	Geometry	Adsorption
Vs/Vv (ml)	0.5	Variable	0.5
Vv (ml)	20	Variable	20
pH	8.5	8.5	Variable
Adsorption temperature (°C)	40	40	Variable
Adsorption time (min)	240	240	Variable

[a] Saturated NaCl, magnetic stirring, desorption at 240 °C, 5 min desorption time.

2.3. Experimental design

Among all the parameters affecting the extraction quality, most have identical values in the published SPME methods. This is the case with salt concentration, which is commonly sodium chloride at saturation, and with magnetic stirring. In the same way, a compromise between desorption time and temperature should be achieved in order to avoid carry-over. Therefore, only five parameters have to be optimised: vial volume (Vv), ratio between the sample volume and vial volume (Vs/Vv), sample pH, adsorption temperature and adsorption time. Instead of studying the five parameters together, this was simplified by dividing them into two groups, by grouping the interdependent parameters.

Vv and Vs/Vv were evaluated together using a two-factor full factorial experimental design. They define the geometry of the headspace but do not facilitate molecules to pass from aqueous phase to the headspace. Vial volume choice was restricted to 20 and 50 ml. These volumes are different enough and contain a reasonable sample quantity. Vs/Vv adviced by the constructor is ca. 75%. This value and a smaller one were selected. Table 2 shows the experimental domain. The matrix of the experiments is presented in Table 3.

Table 2
Experimental domain for "geometry" experimental design

Factor	Parameter	Level −1	Level +1
$X1$	Vial volume (ml)	20	50
$X2$	Sample volume/vial volume	0.5	0.75

Table 3
Two-factors full factorial experimental design

Experiment	$X1$	$X2$
1	−1	−1
2	+1	−1
3	−1	+1
4	+1	+1

Table 4
Three-factors Doelhert experimental design (13 assays plus 2 centre repetitions)

Experiment	$X1$	$X2$	$X3$
1	0	0	0
2	1	0	0
3	0.5	0.866	0
4	−0.5	0.866	0
5	−1	0	0
6	−0.5	−0.866	0
7	0.5	−0.866	0
8	0.5	0.289	0.816
9	−0.5	0.289	0.816
10	0	−0.577	0.816
11	0.5	−0.289	−0.816
12	−0.5	−0.289	−0.816
13	0	0.577	−0.816
14	0	0	0
15	0	0	0

After the "geometry" experimental design, a second experimental design was planned to evaluate the last three parameters. A three-factor Doelhert design was used and 2 centre points were added to ensure enough degrees of freedom for experimental error evaluation (Table 4). After attribution of parameters to factors according to the number of levels, the experimental domain was determined and listed in Table 5.

Each design was tested with the same fibre on a wine model solution spiked with IBMP at 1 $\mu g\,l^{-1}$. Quality evaluation was performed through a GC-NPD analysis, by measuring of IBMP peak area. The higher the peak area, the better is the extraction. Data analysis was performed by means of the statistical software Mathcad® (Mathsoft, Cambridge, UK).

2.4. GC analysis

2.4.1. HS-SPME-GC-NPD analysis for HS-SPME optimisation

Since, under same experimental conditions, [$^2H_2$]-IBMP behaved similarly to IBMP, this study was restricted to IBMP only. Chromatographic analysis was performed with a Trace 2000 (Thermofinnigan) equipped with a nitrogen–phosphorus detector (NPD) system. The NPD temperature was 300 °C. The make-up gas was nitrogen flowing at 15 ml min^{-1}. The source current was adjusted to 13 pA and the polarisation tension was fixed at 3.5 V.

Table 5
Experimental domain for Dolhert experimental design

Factor	Parameter	Level −1	Level 0	Level +1
$X1$	Adsorption time (min)	60	180	300
$X2$	Adsorption temperature (°C)	30	55	80
$X3$	pH	6	7.5	9

Detector gas flows were: 1.7 ml min^{-1} H_2, 15 ml min^{-1} N_2 and 60 ml min^{-1} air. The carrier gas was hydrogen flowing at 1 ml min^{-1}. The injection was done in the splitless mode for 3 min at 240 °C.

The oven temperature was programmed as follows: 50 °C (3 min), then a first ramp (40 °C min^{-1}) to 90 °C, then a second ramp (4 °C min^{-1}) to 140 °C. The final temperature was raised to 230 °C at 20 °C min^{-1} and was maintained for 10 min.

A BPX5 (30 m × 0.32 mm × 0.25 μm) capillary column was used. Data handling was performed by Chromquest software (Thermofinnigan, Courtaboeuf, France).

2.4.2. HS-SPME-GC-MS/MS for analysis

Analysis was carried out using a Varian Saturn 2000 gas chromatograph-ion trap mass spectrometer (GC-ITMS). A DBWAX-ETR (30 m × 0.25 mm × 0.5 μm) capillary column was used. Desorption was realised in the splitless mode over 5 min at 240 °C, with CO_2 cryogenic focussing at the top of the column.

The oven temperature program is the same as already described.

The carrier gas was helium N60 flowing at 1.0 ml min^{-1}. The temperatures of the transfer line and ion trap were 170 and 150 °C, respectively. Detection was performed by electron impact (EI) MS/MS in the multiple reactions monitoring mode, with the following parameters: scan channel 1, parent ion m/z 124 with an isolation window of 1 amu in non-resonant mode, excitation storage 40.7 m/z, excitation amplitude of 38 eV; scan channel 2, parent ion m/z 126 with an isolation window of 1 amu in non-resonant mode, excitation storage 41.4 m/z, excitation amplitude 40 eV. Natural and deuterated IBMP were quantified using the ions m/z 95 and 97, respectively. The ions m/z 81, 109, 124 and m/z 81, 109, 126 were used as qualifiers.

2.5. Wine samples

Fer servadou experimental wines were made at the experimental cellar of ITV at Gaillac. They are derived from different plots and from different harvest dates. All samples were stored in dark bottles at +4 °C until analysed.

3. Results and discussion

3.1. Experimental design

3.1.1. Geometric factors

Two vial volumes were tested: 50 and 20 ml. Ratios Vs/Vv of 75 and 50% were also tested. The results are shown in Fig. 1. No modelling was sought. The highest response was tracked and tested to show if it was significantly different from the others.

By means of the Student's t-test and standard deviation ($n = 5$ on assay 1), the equivalence between answers Y1,

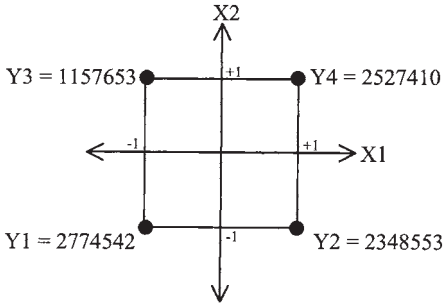

Fig. 1. "Geometry" experimental design results (Yi response = IBMP peak area).

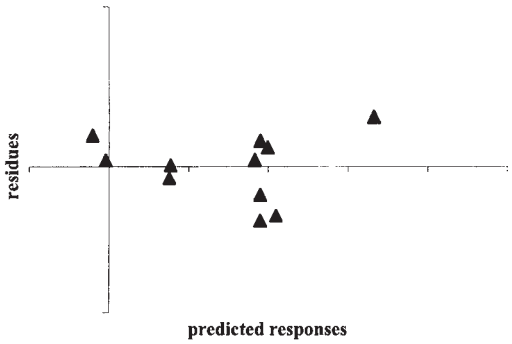

Fig. 2. Residues analysis. Evaluation of model adjustment. Points are well distributed on both sides.

the highest response, and Y2 and Y4, was established. The poor extraction of assay 3 can be explained by the inadequate configuration of extraction: a long and narrow vial compared to a large volume of sample. Magnetic stirring was not sufficient to homogenise the sample and the headspace was too confined. Among assays 1, 2 and 4, few sample quantity consumer conditions were retained. So extraction will proceed in a 20 ml vial sample filled to 50%.

3.1.2. Adsorption factors

Rising temperatures defined the order of experiments. Data underlined that the temperature domain was too high; in fact the IBMP peak area decreased strongly when the temperature exceeded 48 °C. This can be explained by the thermolability of the molecule and by preferential molecule transfer from fibre to headspace gas phase. That is the reason why experiments 8 and 9 were carried out at 27 °C instead of 62 °C as expected. The results are presented in Table 6.

The area prediction calculated model is given by the following relation: area $= 949\,503 - 264\,033X1 - 3\,408\,018 - 1\,327\,344X3 - 154\,891X1X2 - 333\,265X1X3 - 1\,414\,976X2X3 - 299\,017X1^2 + 3\,006\,790X2^2 - 2\,759\,914X3^2$.

Table 6
Adjusted matrix of Doelhert experimental design presenting results in realisation order

Experiment	X1	X2	X3	IBMP peak area
7	4	33	6.5	6328963
6	2	33	6.5	6022206
5	1	55	6.5	936037
1	3	55	6.5	764607
13	3	69	4.5	0
2	5	55	6.5	390160
14	3	55	6.5	853207
11	4	48	4.5	881251
12	2	48	4.5	1059535
3	4	77	6.5	0
4	2	77	6.5	337290
8	4	27	8.5	6455943
9	2	27	8.5	7049401
10	3	41	8.5	1828640
15	3	55	6.5	1035853

Coefficient uncertainties: ±126008; ±124190; ±127481; ±225742; ±222489; ±310260; ±393057; ±206225; ±194143; ±310185.

Several indicators were used to evaluate the model adjustment: R^2 (0.9972), adjusted R^2 (0.9922) and residues analysis (Fig. 2). These values shown that the lack of adjustment is not too large. The model represents satisfactorily the IBMP peak area variations as a function of temperature, pH and adsorption time.

Next, canonical analysis and iso–response curves allowed the optimum conditions to be found. Firstly, stationary point co-ordinates were calculated (−0.38; +0.48; −0.34). This point belongs to the experimental domain, thus an RT-type canonical analysis will be performed. The results of the analysis permit the conclusion that a pH ca. 6.6, 165 min adsorption time at 27 °C are the optimal conditions. Compared to previous SPME extractions [4,7], the adsorption time has been considerably reduced without introducing any fastidious and time-consuming sample preparation step.

3.2. Validation

For calibration, linear regression analysis of relative areas versus relative concentrations of [$^2H_2$]-IBMP and IBMP was used. The calibration equation based on 5 points (3 replicated) is: $\text{area}_{\text{IBMP}}/\text{area}_{[^2H_2]\text{-IBMP}} = 2.02 \times [\text{IBMP}] + 0.71$ ($r^2 = 0.9884$, $n = 15$).

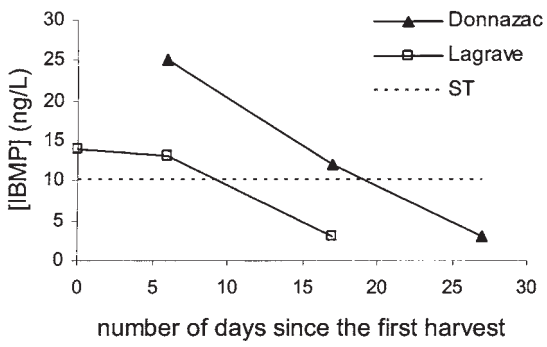

Fig. 3. IBMP amount in Fer servadou wines obtained from different harvest dates.

Table 7
IBMP amount in Fer servadou wines obtained from plots where different techniques have been used

Techniques	[IBMP] ($ng\,l^{-1}$)
Control sample	14
Thin out the leaves under grapes	Not detected
Prior-fermentation maceration at 70 °C	5
Reflective carpet	5

The limit of detection (LOD) was estimated from the minimum concentration found in analysed wines. The LOD was calculated on the basis of a signal/noise ratio of 3. The LOD is $1\,ng\,l^{-1}$, which is far below the sensory threshold. More accurate precision is not needed. The repeatability (4%) was established from 5 repetitions of an analysis.

3.2.1. Analysis of wine samples

Some Fer servadou wines have been analysed by the new method. Harvest date influence (Fig. 3) and viticultural techniques (Table 7) impact on the IBMP amount measured. With a late harvest date the IBMP concentration decrease and can be lower than the sensory threshold. With a late harvest date the IBMP concentration can be lower than the sensory threshold. Therefore, it is interesting not to harvest too early. Furthermore, plots do not have the same "IBMP potential", it depends on soil and exposure. A sandy soil (Lagrave) will warm up quickly and reflect to the grapes thermal and luminous energy. This phenomenon favours IBMP degradation. To reflect soil IBMP potential, it would be interesting to study Fer servadou plots. It could be used to classify the plots according to their IBMP potential and could influence the plots harvest order. Finally, results confirm the efficiency of the viticultural (preventive) and enological (curative) techniques used.

4. Conclusions

The method's sensitivity for detection of IBMP is far below the sensory threshold. Moreover the repeatability is lower than expected for SPME extraction (CV ca. 12%), this by the use of stable isotope dilution assay. This latter method seems to fit very well this kind of extraction. The solvent-free method developed will be used in a semi-routine way. It has a lot of applications and will, for example, be very useful to determine the best harvest date according to aroma maturity.

Acknowledgements

This work has been funded by the "Station régionale ITV Midi-Pyrénées" (Gaillac, France). The authors are also grateful to UNICOR and Thermofinnigan for their support.

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